REMARKS

Claims 43-72 were pending in the present application. Of these, claims 62 and 66 have been withdrawn by the Examiner as allegedly directed to non-elected subject matter. By this Amendment, Applicants have canceled claims 43-72 without prejudice and have presented new claims 73-102. Support for the new claims can be found throughout the specification and claims as originally filed. The present Amendment introduces no new matter, and thus, its entry is requested. Upon entry of the present Amendment, claims 73-102 will be pending and under examination.

Information Disclosure Statement

The Examiner indicated that the Information Disclosure Statement filed April 8, 2002 is in compliance with the provisions of 37 C.F.R. 1.97 and the references included therein have been considered. The Examiner noted, however, that the submitted copy of the Ye reference appeared to be incomplete. Applicants therefore are submitting herewith a new copy of the previously submitted Ye reference.

Examiner's Rejections under 35 U.S.C. §112

The Examiner rejected claims 43-61, 63-65, and 67-72 under 35 U.S.C. 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim

the subject matter which applicant regards as the invention. The Examiner indicated that the rejection is primarily concerned with the language of claim 51, which describes the method of claim 43 wherein the immobilization comprises the adsorption onto a metal modified crystal. Specifically, the Examiner indicated that claim 51's reference to adsorption onto a "metal modified" crystal is unclear. The Examiner questioned whether the term "metal modified" includes or is in addition to the attachment of the electrodes to the Pz crystal. According to the Examiner, if the former is the case, the claims would appear to read on methods of performing the assay wherein the Pz crystal has not been attached to electrodes, which electrodes are necessary to induce an electric field. In other words, the implication of claim 51's delimiting an embodiment in which the crystal is metal modified is that the broader method of claim 43 can also therefore be performed using a crystal which is <u>not</u> metal modified. According to the Examiner, it is not apparent how the method would operate if the Pz crystal is not metal modified. In the alternative, if the claims are construed to include metal modification that is something other than the attachment of the metal electrodes, it is the Examiner's position that the scope of such modification is not clear. According to the Examiner, the application discloses adsorption onto the bare metal electrodes, but offers no discussion of metal modification other than the attachment of the electrodes to the crystal with subsequent attachment of the antigen/antibody to the electrodes. The Examiner has thus concluded that there is insufficient description of what constitutes "metal modification."

The Examiner also rejected claims 43-61, 63-65 and 67-72 under 35 U.S.C. §112, first paragraph, for lacking enablement. According to the Examiner, there is insufficient enablement for the claimed methods in which the Pz crystals are not metal modified. The rationale for this rejection is essentially the same as that set forth in connection with the above indefiniteness rejection. In particular, the Examiner asserted that the art (e.g. Bastiaans et al., U.S. Patent 4,735,906, of record in the April 2002 IDS; and Larue, U.S. Patent 5,705,399) and the specification (pages 2-5) teach that the metal electrodes attached to the crystal are necessary for the claimed method to operate because these electrodes provide the energy required to induce the resonance of the crystal, which in turn is required to make the readings necessary for the claimed immunodiagnostic test operable. Thus, in the Examiner's opinion, the claimed methods or kits wherein the Pz crystals are not metal modified are not enabled by the specification.

In response, without conceding the correctness of the Examiner's position, but to expedite allowance of the subject application, Applicants have presented new claims which do not include the language which forms the basis of the Examiner's indefiniteness and enablement rejections. Specifically, new claim 82 (which corresponds to canceled claim 51) now recites "1) physical adsorption onto a bare metal electrode, 2) physical adsorption onto a hydrophobic polymer modified crystal, and 3) covalent binding onto a silane or thiol compound modified crystal." The language of new claim 82 is consistent with, and thus supported by, *inter alia*, page 11, lines 13-16, of the specification. Therefore, in response to the Examiner's remarks, on page 4, claim 51's reference to a "metal modified" crystal was with respect to attachment to the bare metal

electrode. The new claims fully comply with the requirements of 35 U.S.C. §112, and thus Applicants respectfully request that the indefiniteness and enablement rejections be withdrawn.

Examiner's rejections under 35 U.S.C. §103

The Examiner also set forth various rejections under 35 U.S.C. §103(a) of all pending claims. The Examiner's detailed rationales for these rejections are set forth at pages 5-10 of the Office Action. The primary reference cited in each of these rejections is Bastiaans, et al. (U.S. Patent No. 4,735,906), which the Examiner noted is already of record as a reference included in the previously submitted Information Disclosure Statement. The Examiner cited the additional references Larue (U.S. Patent No. 5,705,399), Thorns (U.S. Patent No. 5,510,241), Rajashekara, et al. (WO 98/03656), Willner, et al. (WO 98/40739), Willner, et al. (WO 98/04314), Masten, et al. (J. Bacteriol 175: 5359-65), and CAA78777 in the various rejections, for their teachings of features not supplied by the Bastiaans, et al. reference.

According to the Examiner, Bastiaans teaches the use of Pz crystals in immunodiagnostic tests. (The Examiner directed attention to columns 1-3). In the Examiner's opinion, the reference teaches methods that are substantially similar to those used in the present application in that one compound of a binding pair (e.g. an antibody or antigen) is bound to the crystal, which is then exposed to a sample to detect the presence of the binding partner. The Examiner asserted that the reference further teaches that used crystals may be cleaned and reused, and that the Pz crystal resonance measurements may be made using a universal counter. (Column 4, lines 7-11,

and 43-46). According to the Examiner, similar teachings are also provided in Larue (abstract, columns 1-5), which the Examiner stated also suggests the use of the Pz devices for the detection of antigens of illnesses, including those caused by Salmonella infections. Column 11, lines 11-27.) The Examiner further asserted that each of these references (Bastiaans and Larue) also teaches that, after use, the devices may be cleared of bound antibody/antigen using compounds with high ionic strength (including sodium chloride solutions), or low pH.

In response, without conceding the correctness of the Examiner's positions, but to expedite allowance of the subject application, Applicants have presented new claims 73-102, which Applicants believe fully overcome all of the Examiner's rejections under 35 U.S.C. §103. Applicants point out that the newly presented method and kit claims recite, *inter alia*, that the PZ crystal that is used in the assay was previously used in a test which was negative for the infectious agent associated with the disease. None of the art cited in the present Office Action, either alone or in combination, teaches or suggests a method of detecting veterinary diseases in an animal with such features. Although the Bastiaans and Larue references refer to washing and regenerating crystals for further use after obtaining a positive result, there is no disclosure of reusing crystals already tested against negative samples, and thus no recognition of the advantages for veterinary applications of methods having this feature. Some of these advantages are described on page 6, lines 21-26 of the specification and include the possibility of rapid on-site testing of samples and the real-time display of results. These, among others, are valuable advantages in the field of veterinary medicine where previously it would have been necessary to

take samples on-site and transfer them to a suitably equipped laboratory for a traditional immunoassay. Not only does the invention provide a more convenient and simple procedure for the diagnosis of disease in animals, but it also allows for a much faster diagnosis. This can be vital in helping to prevent the spread of disease and the suffering of the animals.

A further advantage is that the method of diagnosis according to the invention has a fundamental low cost. One factor that contributes to this cost-effectiveness is the reusability of the sensors after they have been used to detect a negative serum. Page 28, lines 14-26 of the specification describes the advantages of this feature. Although the cited art, in particular the Bastiaans patent, refers to the reuse of sensors after coming into contact with positive samples (after chemically treating the sensor to remove bound protein), it does not recognize the advantages of simply reusing a sensor after it has come into contact with a negative sample. The present invention thus provides significant advantages for testing for veterinary diseases in animals, none of which advantages are taught or suggested by the cited art, alone or in combination.

In view of the above amendments and remarks, it is believed that the claims satisfy the requirements of the patent statutes and fully address the Examiner's concerns as set forth in the March 25, 2004 Office Action. Reconsideration of the instant application and early notice of allowance therefore are requested. The Examiner is invited to telephone the undersigned if it is

deemed to expedite allowance of the application.

Respectfully submitted,

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Piezoelectric Biosensor for Detection of *Salmonella typhimurium*

JIANMING YE, STEPHEN V. LETCHER, and ARTHUR G. RAND

ABSTRACT

A flow injection analysis (FIA) system was developed based on a piezoelectric biosensor for detection of *Salmonella typhimurium*. The anti-*Salmonella* spp antibody was immobilized onto the gold electrode coated quartz crystal surface through a polyethylenimine-glutaraldehyde (PEG) technique and dithiobis-succinimidyl propionate (DSP) coupling. The PEG technique proved more successful for FIA applications than the DSP coupling. The biosensor had responses of 23–47 Hz in 25 min when the PEG immobilization technique was employed, with $R^2>0.94$ for *Salmonella typhimurium* concentrations of 5.3×10^5 to 1.2×10^9 CFU/mL.

Key Words: piezoelectric, biosensor, *Salmonella*, antibody immobilization, QCM

INTRODUCTION

THE REPORTED INCIDENCE of *Salmonella* infections from food in the United States has increased substantially since reporting began in 1943 (Tauxe, 1991; CAST, 1994). According to the Center for Disease Control (CDC), during 1983-1987, there were 342 reported salmonellosis outbreaks, 31,245 reported cases, and 39 reported deaths (Bean et al., 1988). This accounted for 34.5% of the reported foodborne disease outbreaks, 57.3% of the reported cases, and 28.5% of reported deaths. The small amount of *Salmonella* found in food, especially in comparison to the larger number of other organisms makes the presence of *Salmonella* unlikely to be detected by direct plating on selective media, and an enrichment procedure in broth is needed. Since most foods assayed for *Salmonella* are very perishable, the time for detection is limited. Therefore, rapid and precise methods for *Salmonella* detection are needed.

Traditional cultural methods are very slow and costly, requiring 3–4 days for presumptive results and 5–7 days for confirmation of *Salmonella* (Rodrigues and Kroll, 1990). Many attempts have been made to accelerate, simplify and improve the reliability of detection methods. Among these are the fluorescent antibody test (Rodrigues and Kroll, 1990; Tsai and Slavik, 1994), various enzyme-linked antibody tests (Minnich et al., 1982; Flowers et al., 1988), DNA hybridization methods (Fitts et al., 1983) and improved culturing methods(Poppe and Duncan, 1996). Most such tests have shown considerable degrees of success, but most require about 8–10 hr from initial sampling to final results.

One approach that has received increasing attention is a piezoelectric biosensor (Prusak-Sochaczewski and Luong, 1990a, b), which combines the specificity of antibodies with the high sensitivity of a quartz crystal microbalance (QCM). A vibrating quartz crystal would be an extremely sensitive weight indicator, since the frequency of the crystal could be accurately measured. A crystal controlled oscillator becomes a promising highly sensitive monitor of anything that changes the resonant frequency of the piezoelectric biosensor, especially mass change (Luong and Guilbault, 1991). Antibodies attached to the crystal should be able to detect the specific antigen that combines with the antibody with extreme specificity and sensitivity (Prusak-Sochaczewski and Luong, 1990a, b; Prusak-Sochaczewski et al. 1990; Mimunni, 1994).

Two immobilization techniques show promise to immobilize anti-*Salmonella* spp antibody onto gold electrode coated quartz crystal surfaces. The first is dithiobis-succinimidyl propionate (DSP) coupling. The bifunctional reagent DSP is widely used for binding protein molecules via amino groups (Hermanson et al., 1992). In addition to succinimidyl groups, DSP has a disulfide linkage that will rapidly chemisorb to gold surfaces with a resulting stability exceeding that of covalent saline bonds with glass (Hermanson et al., 1992; Katz, 1990). Another method was described by Santos (1994) for enzyme immobilization. The crystal is pre-coated with a thin layer of polyethyleneimine and activated with glutaraldehyde. Proteins like enzymes and antibodies could then attach to the surface through amino groups.

A piezoelectric crystal with immobilized anti-*Salmonella* spp antibody should react specifically with *Salmonella* cells and bind them to the crystal surface. With a specific amount of antibody immobilized, the cell mass binding to the crystal should be directly related to the concentration in the solution. In previous studies, the frequency of the crystal has been measured in the dry state, which is rather cumbersome and time consuming. A more efficient analysis system is needed to improve the accuracy, reliability and rapidity.

A biosensor system combining the antibody coated piezoelectric crystal with a stable, easy-to-use flow injection analysis (FIA) system has the potential for real time detection of *Salmonella* within minutes. Therefore, the objectives of this study were to utilize antibody immobilization techniques on gold-coated quartz crystal surfaces, develop a FIA piezoelectric biosensor system, and test immobilized antibody in such a FIA system for rapid *Salmonella* detection.

MATERIALS & METHODS

Materials

Acetone and potassium phosphate [monobasic (pH 4.3) and dibasic (pH 9.5)] were obtained from Fisher Scientific Co. (Springfield, NJ). Glutaraldehyde (GA), 50% in water, was purchased from Eastman Kodak Co. (Rochester, NY). Dithiobis-succinimidyl propionate (DSP), polyethyleneimine (PEI), 50% w/v aqueous solution, and Bovine serum albumin (BSA), protein standard solution 10g/dL, were obtained from Sigma Chemical Co. (St. Louis, MO). Bio-Rad protein assay dye reagent concentrate and IgG standard were obtained from Bio-Rad Laboratories (Melville, NY). Anti-*Salmonella* spp antibody (around 4–5 mg/mL) was purchased from Biodesign International (Kennebunk, ME.). *Salmonella typhimurium* (ATCC 13311) was purchased from American Type Culture Collection (Rockville, MD.) and confirmed by positive TSI reaction and negative urease test (FDA, 1984) and maintained in nutrient broth. Microcon-100 microconcentrators were obtained from Amicon, Inc. (Beverly, MA.). AT-cut piezoelectric quartz crystals, 1.27cm in diameter, with vapor deposited coaxial gold electrodes (1.11cm/0.95 cm) and a fundamental frequency of about 5 MHz, were purchased from Valpey Fisher (Hopkinton, MA). The oscillator circuit was built by the Physics Department of the University of Rhode Island. The polypropylene flow cell was designed and built in our lab. The septum injector was purchased from Rainin Instruments Co.(Woburn, MA).

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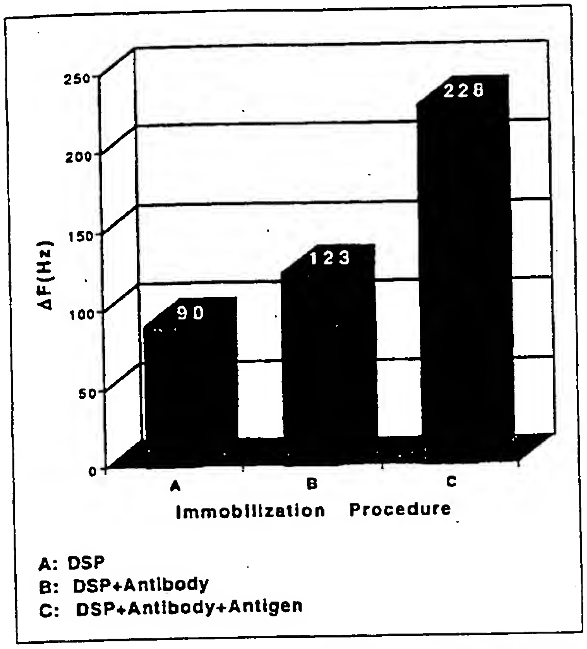

Fig. 1—*Salmonella* piezoelectric biosensor dry measurement of the frequency changes (ΔF) at each step of DSP coupling for anti-*Salmonella* spp antibody attachment and antigen binding to quartz crystals.

Bacteria culture handling

A *Salmonella typhimurium* culture, which had been growing in nutrient broth at 37°C for about 8 hr and had a viable bacteria count of 10^8 CFU/mL was used as starter culture. This culture was maintained in cryogenic vials and stored at −70°C. For each experiment, a cryogenic vial was revived to prepare the new culture. The culture growing in nutrient broth at 37°C was diluted and spread on nutrient agar plates. The plates were incubated at 37°C for 24 hr to determine viable cell counts.

Preparation of concentrated anti-*Salmonella* spp antibody

The anti-*Salmonella* spp antibody was concentrated with an Amicon Microcon-100 microconcentrator. These microconcentrators employed the low-binding, anisotropic, hydrophilic YM membrane. First, the sample reservoir was put into the vial above the membrane. Then, 200 µL of the original antibody solution was pipetted into the reservoir and centrifuged at 3,000 × *g* for about 10 min to reduce the volume to almost half. The sample reservoir was then placed upside down in a new vial and centrifuged at 1,000 × *g* for 3 min to transfer the concentrate to the new vial. Concentrations of the original and concentrated antibody solutions were measured by the Bio-Rad Protein Assay.

Immobilization procedures

Immobilization through DSP coupling was achieved with the quartz crystal starting with a clean gold surface, which was first washed with deionized water, soaked in 1.0N NaOH for 20 min followed by 5 min in a 1.0N HCl treatment. The crystal was then washed with deionized water, 95% ethanol and air dried. The crystal was immersed into a 0.01M dithiobis-succinimidyl propionate (DSP) acetone solution at room temperature for 1 hr. After incubation, the crystal was thoroughly rinsed with fresh acetone solution and air dried. About 15 µL of anti-*Salmonella* spp antibody solution was applied onto the crystal surface and incubated in a wet chamber containing deionized water at 4°C overnight. After being rinsed with 0.05M phosphate buffer, the crystal was immersed for 3–4 hr at 4°C in a 100 mg/mL BSA (bovine serum album) solution to block all unreacted sites. The crystal was then washed and immersed in 0.05M phosphate buffer, pH 7.2, and stored at 4°C. Each step of immobilization with DSP coupling was monitored with the dry assay procedure.

Immobilization via polyethyleneimine-glutaraldehyde (PEI-GA) layer was also conducted after the piezoelectric crystal was cleaned as described and then 3 µL of a methanol solution containing 2% PEI was dispersed onto the surface of the electrode and the crystal was air dried. The crystal was then immersed in a 2.5% GA solution for 30 min, and air dried. About 15 µL of anti-*Salmonella* spp antibody solution was applied over the crystal surface. Incubation was carried out in a wet chamber containing deionized water overnight at 4°C. The unreacted sites were blocked with BSA for 3–4 hr at 4°C. Then the crystal was stored in 0.05M phosphate buffer pH 7.2 at 4°C for later use.

Reference crystals for each of the immobilization methods were prepared the same way as sensing crystals but without attaching the antibodies.

Measurement procedures

A dry measurement procedure for *S. typhimurium* was used in the initial stages to monitor the immobilization procedure. Six different crystals were used for each bacteria concentration, three as sensing crystals and three as reference crystals. The crystal was clamped between two metal wires connected to the oscillator which was monitored by the Philips 6666 frequency counter. The fundamental frequency of the crystal was first determined as F1, and then the crystal was dipped into bacterial cell suspensions containing 10^5–10^8 CFU/mL for 30 min at room temperature. The crystal was washed with phosphate buffer and air dried. The crystal frequency change due to *Salmonella* cells attached to the crystal surface was tested as a new value F2. The frequency decrease of the crystal was reported as ΔF_s ($\Delta F_s = F1_s - F2_s$) for the sensing biosensor, or ΔF_r ($\Delta F_r = F1_r - F2_r$) for the reference. The frequency changes of reference crystals (ΔF_r) were < 3 Hz. The actual frequency decreases ΔF were calculated ($\Delta F = \Delta F_s - \Delta F_r$) and the average decrease in frequency for three crystals was related to the concentration of *S. typhimurium* cells.

In the FIA of *S. typhimurium*, five different sensing crystals and two reference crystals were prepared for each bacteria concentration. The reference crystal was used at the beginning and the end of each assay. The antibody coated crystal was mounted inside a flow cell and 0.05M phosphate buffer, pH 7.2, was pumped through for use as a FIA system. The buffer flow rate was 0.15 mL/min. After stabilization of the oscillation frequency, 60 µL of a *S. typhimurium* cell suspension (10^5 CFU/mL-10^9 CFU/mL) was injected and pumped into the flow cell. To allow better interaction between antibodies and cells, the pump was stopped 1 min after injection and the frequency base-line was recorded as F1. After 25 min of antibody-cell interaction, another frequency was read as F2. The crystal was then replaced with a fresh crystal and the analysis repeated five times. The frequency decrease of the crystal was reported as ΔF_s ($\Delta F_s = F1_s - F2_s$) for the sensing biosensor or ΔF_r ($\Delta F_r = F1_r - F2_r$) for the reference. The average frequency decrease of reference crystals was <5 Hz and was used as background signal. The actual frequency decrease ΔF was calculated by subtracting the background signal from the frequency change of the biosensor and the average decrease in frequency of five sensing crystals was related to the concentration of the injected *S. typhimurium* cells.

Statistical analysis

All analyses were reported as means of 3 or 5 replicates. The standard deviation was determined for each mean. Data on graphs with error bars are mean values ± one standard deviation (M ± S.D.). The relationship between ΔF and bacterial cell concentration was determined by linear correlation plots.

RESULTS & DISCUSSION

THE IMMOBILIZATION PROCEDURE for the anti-*Salmonella* spp antibody was monitored using a dry assay to measure the frequency change at each step of DSP immobilization and reaction with *S. typhimurium* cells. Frequency changes typically occurred (Fig. 1) at each step of immobilization in the dry assay. When DSP was coupled to the electrode, the frequency decreased 90 Hz. There was a further 33 Hz decrease when anti-*Salmonella* spp antibody was attached to the DSP layer on the crystal. Then the crystal was allowed to react with *S. typhimurium* cells, and the frequency decreased another 105 Hz. The frequency change permitted an estimate of the material attached and added to each

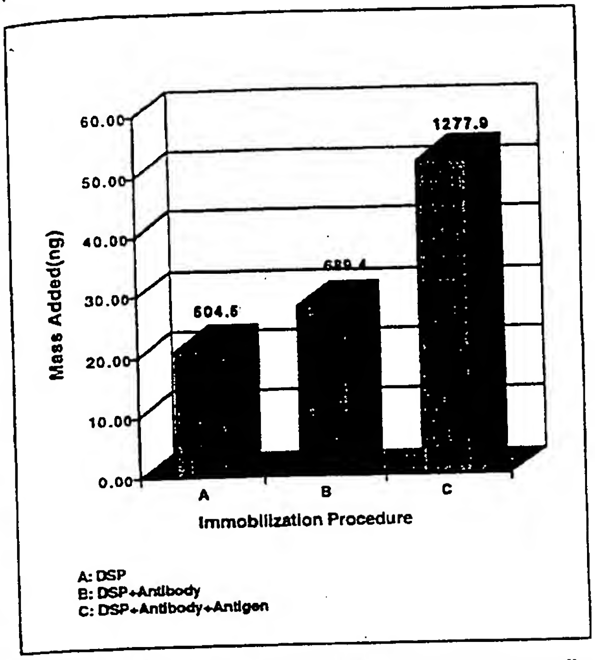

Fig. 2—Calculation of mass loading at each step of DSP coupling for anti-*Salmonella* spp antibody attachment and *Salmonella* binding to piezoelectric quartz crystals employing the dry measurement procedure.

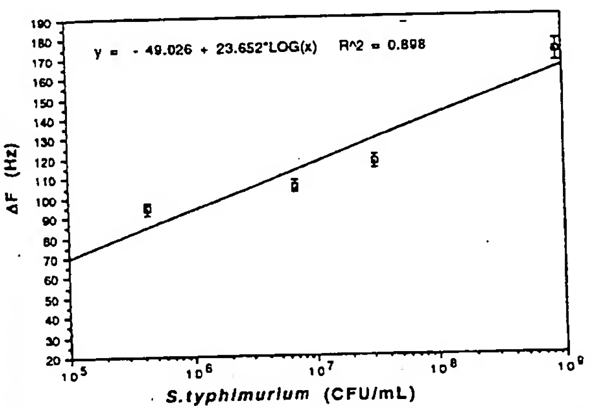

Fig. 3—Piezoelectric biosensor dry assay for *S. typhimurium* with DSP antibody immobilization showing the relationship between the frequency change (ΔF) of the crystal and the concentration of bacterial cells (M ± S.D., n=3).

layer on the surface. The mass added to the crystal was calculated by the Sauerbrey equation (Luong and Guilbault, 1991):

$$\Delta F = -2.26 \times 10^{-6} F^2 \Delta m/A$$

where ΔF was the frequency decrease; F was the resonant frequency; Δm was the mass added; and A was the area of the electrode surface. Based on frequency changes the calculated mass loading on the quartz crystal surface was determined (Fig. 2). The mass added at each step proved to be directly related to the frequency decrease, and changed with each additional attachment stage. These results indicated that the success of attachment and the mass loaded could be predicted and calculated.

The biosensor constructed with DSP was then tested for response to different concentrations of *S. typhimurium* cells (Fig. 3). The response of the biosensor was linear from 10^5–10^9 CFU/mL with ΔF changing from 90-170 Hz. A relatively high correlation coefficient(R^2=0.898) was found. Therefore, DSP was a successful immobilization method to construct a biosensor for dry assay procedures.

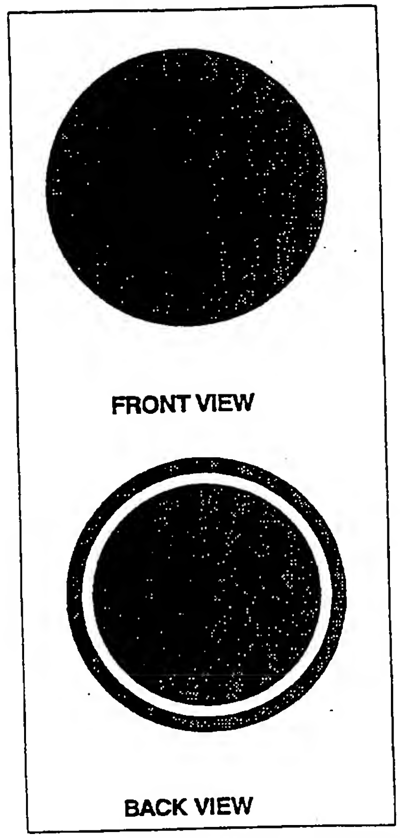

Fig. 4—Front and back views of a coaxial 5 MHz AT-cut piezoelectric quartz crystal with gold electrodes.

Piezoelectric biosensors utilizing the DSP immobilization method were then incorporated into a flow injection analysis (FIA) system. One side (front) of the coaxial quartz crystal was fully covered with a gold electrode which wrapped around to the other side (Fig. 4). The back side had a central circular gold spot of 0.95 cm diameter and an outer gold deposited ring which was part of the opposite side electrode. Both electrode connections were made on the back side of the crystal, while the antibodies were immobilized on the front side. The sensing crystal with the antibody-immobilized surface mounted face-up was clamped between two rubber O-rings in a flow cell designed and built specifically for use with the coaxial type of quartz crystal (Fig. 5). Only one side of each crystal was exposed to the solution. A reference crystal without immobilized antibody was used to eliminate the effect of nonspecific adsorption. The outer gold electrode was connected to the plug directly through an aluminum wire, and the center electrode through an aluminum wire connected to a small spring. A septum injector was installed on top of the flow cell which allowed the sample to be injected into the buffer stream close to the inlet of the flow cell. The piezoelectric biosensor flow cell was incorporated into a total FIA system (Fig. 6). The flow cell was plugged directly into a crystal-control oscillator powered at 5-volts by a LPS-300 series linear DC power supply. The output of the oscillator was monitored by a Phillips PM 6666 frequency counter. A Power Macintosh computer with the LabVIEW software was used to collect and analyze data from the frequency counter. The 0.05M phosphate buffer, pH 7.2, was pumped through the flow cell by a Cole-Parmer Masterflex L/S Peristaltic pump.

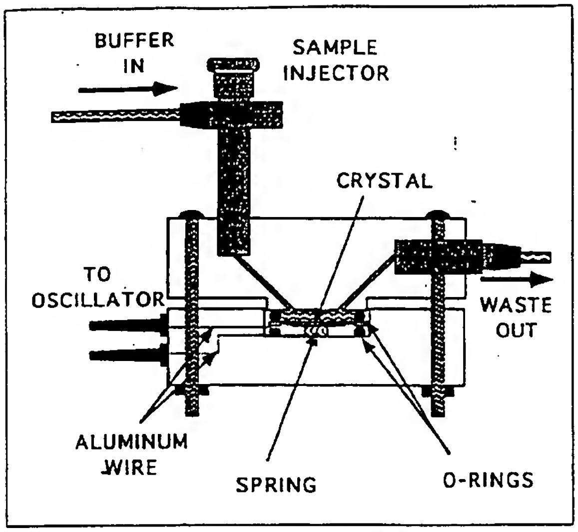

Fig. 5—Piezoelectric biosensor flow cell design.

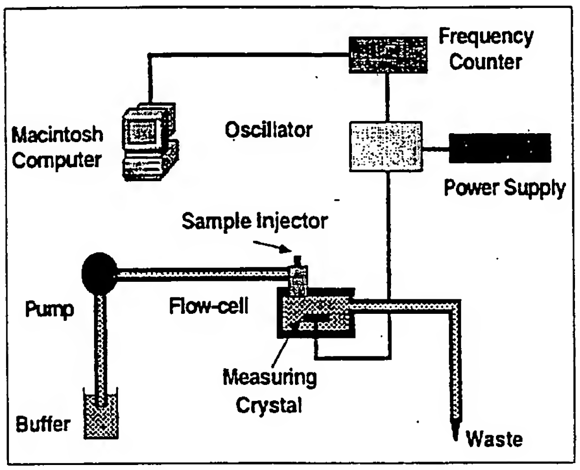

Fig. 6—Schematic of flow injection analysis piezoelectric biosensor system.

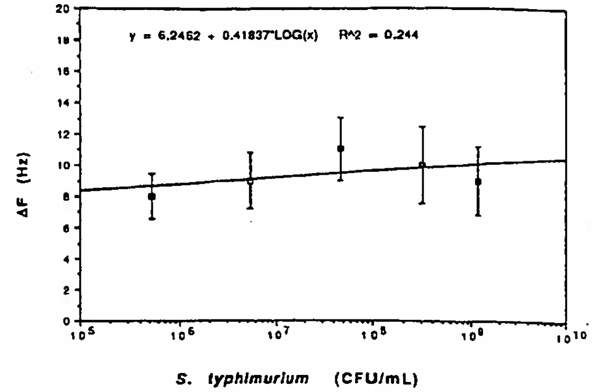

Fig. 7—*Salmonella* piezoelectric biosensor with DSP antibody immobilization method showing the correlation of frequency change (ΔF) and cell concentration in flow injection analysis at room temperature, pH 7.2 (M ± S.D., n=5).

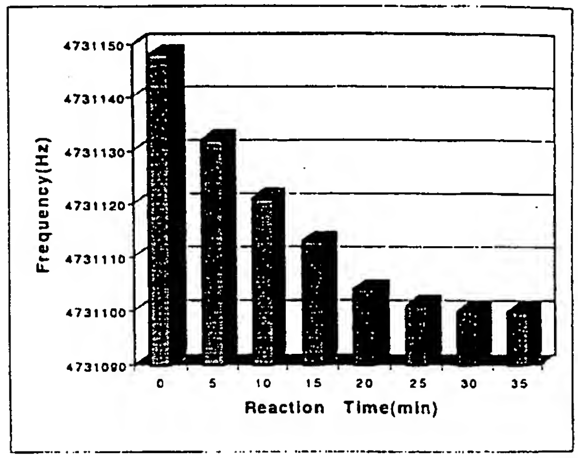

Fig. 8—*Salmonella* biosensor with PEI-GA antibody immobilization method showing the relationship between frequency change and stop flow reaction time in flow injection analysis at room temperature, pH 7.2.

The biosensors with DSP-immobilized antibody were tested in the FIA with *S. typhimurium* cell injection (Fig. 7). There was almost no difference in biosensor response (8-11 Hz) to *Salmonella* cell counts ranging from 10^5–10^9 CFU/mL, correlation coefficient 0.224. There were several possible explanations for the lack of cell-antibody interaction and mass loading when compared with that of dry assay. It has been reported that DSP attachment can be susceptible to hydrolysis in an aqueous solution at room temperature (Hermanson et al., 1992). Since the reference crystal had the same response to the same concentration bacterial samples, it appeared that the antibodies were released from the crystal surface and the sensitivity of the sensor decreased to an undetectable level. Also, the crystal was oscillating throughout the reaction in FIA while in dry assay the crystal was active only during the final measurement. The vibration of the crystal and the electrical field might have interfered with the antibody-cell interaction.

Since DSP was not effective in FIA, another immobilization method PEI-GA was tried and the performance of the biosensor constructed with this technique was evaluated. The optimum operating conditions and sample injection volume were first investigated. The best temperature for the quartz sensor was around room temperature. The biosensor system was very stable in oscillating frequency and results were more reproducible when operated at room temperature. The optimum flow rate for the system was determined by operating at different values and monitoring the oscillation stability.

The optimum flow rate in FIA was 0.15 mL/min. At that flow rate, the pulse and the disturbance inside the flow cell were minimized, and the stability of the system improved. The maximum interaction between the immobilized antibody and injected *S. typhimurium* cells was determined by stopping flow for certain times after sample injection. The optimum stop reaction time was determined by monitoring the frequency decrease with time. A typical frequency change of the piezoelectric sensor with time in the FIA system is shown (Fig. 8). The frequency decreased significantly at 5,10,15 and 20 min. The change in frequency from 20 to 25 min was small and after 25 min the frequency change was almost stable. Based on these results, 25 min was chosen as the stop reaction time for further study.

Sample injection volume was also tested to maximize the response of the sensor (Fig. 9). With the same bacterial cell concentration, the frequency change increased about 10 Hz when the injection volume increased 2× from 30 µL to 60 µL. There was no apparent difference between 60 µL and 100 µL injec-

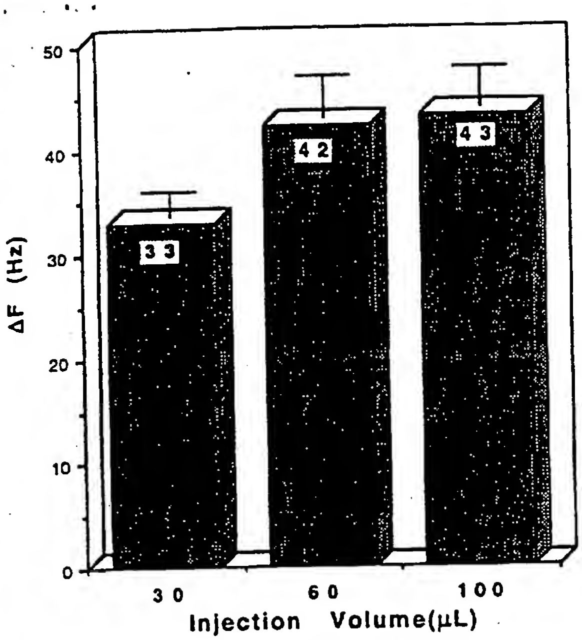

Fig. 9—*Salmonella* biosensor with PEI-GA antibody immobilization method showing the relationship between frequency change (ΔF) and sample injection volume with 2×10^8 CFU/mL *S. typhimurium* cells in FIA at room temperature, pH 7.2 (M ± S.D., n=5).

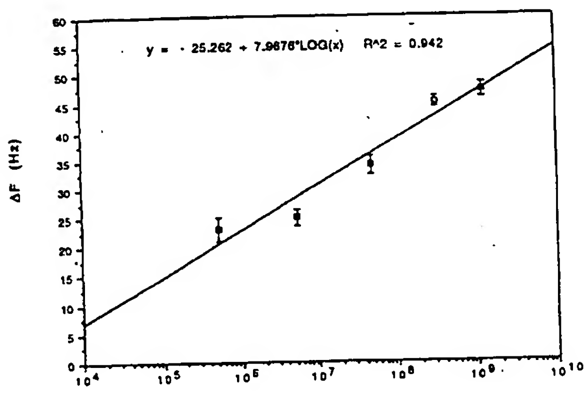

Fig. 10—*Salmonella* piezoelectric biosensor with PEI-GA antibody immobilization method showing the correlation of frequency change (ΔF) and cell concentration in FIA at room temperature, pH 7.2(M ± S.D., n=5).

tions, so 60 μL was selected as the volume of choice for the study.

When the operating conditions of the system were optimized, piezoelectric biosensors constructed with the PEI-GA method were tested in the FIA system. Different concentrations of *S. typhimurium* were injected and the frequency changes were recorded (Fig. 10). The response of the sensor to cell counts ranging from 5.3×10^5—1.2×10^9 CFU/mL was virtually linear and the frequency change ranged from 23 Hz to 47 Hz. Although the sensitivity of the sensor in the FIA was lower in overall F than that of the DSP dry assay, the correlation coefficient improved to 0.942.

The PEI-GA technique was the more reliable immobilization method for FIA operation. The piezoelectric biosensor with the

Table 1—Detection times and sensitivity (without enrichment) of different rapid *Salmonella* methods

Detection method	Detection time	Sensitivity (CFU/mL)
ELISA	hours	10^4-10^5
Fluorescent Immunoassay	hours	10^4-10^5
PCR	hours	10^3-10^4
Fluorogenic Assay	hours	10^3-10^4
Piezoelectric Biosensor	minutes	10^5

Table 2—Response of *Salmonella* biosensor through PEI-GA antibody immobilization using different concentrations of antibody to the same concentration of *S. typhimurium*[a]

	Original antibody[b]	Concentrated antibody[c]	Percentage change[d]
Antibody conc (mg/mL)	5.03±0.4	9.84±0.05	95.6
Biosensor response (Hz)	42±2.9	73±2.2	73.8

[a] 2×10^8 *S. typhimurium* cells per milliliter.
[b,c] Data are M ± S.D. n=3.
[d] Calculated using mean values.

PEI-GA immobilization was very stable in the FIA and the results from the system were reproducible. When compared with other methods (Table 1), the sensor had a very short detection time and a sensitivity close to most other methods without pre-enrichment. The piezoelectric biosensor constructed using PEI-GA technique performed very well in this system. When operated in the FIA system, the bacterial biosensor was very stable, efficient and easy to operate. The combination of an anti-*Salmonella* spp. antibody coated crystal and the FIA system produced a *Salmonella* piezoelectric biosensor with short response time and reasonable sensitivity.

Since the sensitivity of the biosensor in the FIA mode was only comparable to other rapid methods, efforts were made to increase the sensitivity. A key factor which influences sensitivity of any type of piezoelectric biosensor would be the density of immobilized bioactive material. One approach which could improve sensitivity of the piezoelectric biosensor would be to increase the antibody concentration in solutions to be immobilized onto the crystal surface. The use of higher antibody concentration has the potential to produce a much stronger signal to the same cell concentration and improve the detection limit.

In an effort to get higher antibody density onto the crystal surface, the original antibody solution was concentrated by ultrafiltration using a microconcentrator (Amicon). The concentrations of original and concentrated antibody were measured. Piezoelectric biosensors constructed with original and concentrated antibody solutions were tested for their responses to a concentration of 2×10^8 CFU/mL *S. typhimurium* cells. When the concentration of antibody increased 95.6% from 5.03 mg/mL to 9.84 mg/mL (Table 2), the sensor response increased 73.8% from 42 Hz to 73 Hz. Since the linearity of the biosensor with original antibody to different *S. typhimurium* concentrations was established, the signal increase of 73.8% to the same cell concentration by using the concentrated antibody meant that the overall response of the biosensor was increased 40–82 Hz by using concentrated antibody.

The lowest accepted frequency change for this biosensor was 20 Hz, which meant that the detection level for *Salmonella* with concentrated antibody was improved one log cycle to 10^4 CFU/mL. This detection level was close to the most sensitive rapid detection method for *Salmonella* in foods. If this biosensor system could be made reusable, then the potential value of this rapid detection system for *Salmonella* could be maximized. Piezoelectric biosensors have the potential for miniaturization and portability and can be used on samples with minimal preparation.

CONCLUSION

A PIEZOELECTRIC BIOSENSOR SYSTEM showed great potential as a rapid method for detection of *Salmonella*. Anti-*Salmonella* spp

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milk processability due primarily to seasonal changes in animal diet and stage of lactation (Kefford et al., 1995; Phelan et al., 1982). They have also shown variations in milk fatty acid profile between indoor and outdoor feeding (Murphy et al., 1995).

CONCLUSIONS

LEVELS OF CLA can be increased in bovine milk through dietary manipulation. Full-fat rapeseed supplements resulted in substantial increases in CLA in milk, while reduced levels of grass intake caused a reduction in levels of CLA over a 19-wk period. Nutrient composition is a significant contributing factor to CLA levels in milk, but other factors such as lactation number of the animals may contribute.

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antibody immobilized by DSP coupling onto the gold coated surface of quartz crystals performed reasonably well in dry assay conditions, but was not effective in FIA in continuous contact with buffer. The PEI-GA technique was an effective antibody immobilization method on the gold surface of quartz crystals for use in a FIA system which was very stable, efficient and easy to operate. The system produced a piezoelectric biosensor with short response time (25 min) and reasonable sensitivity. The correlation coefficient for frequency change with *S. typhimurium* concentration was 0.942, which indicated good linearity of response. The sensitivity was improved 74% by a 96% increase in the protein concentration of the antibody solution immobilized.

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